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A Set of Oligo-Nucleotides Against HIV Infection and Its Application in the
Prevention and Treatment of Acquired Immune Deficiency Syndrome

Technical Field

5 The invention is regarded to a set of oligo-nucleotides against HIV infection and its application in the prevention and treatment of Acquired Immune Deficiency Syndrome(AIDS).

Technology background

Recent findings proved that short double strand RNA function as
10 interference RNA in a variety of mammalian cells, and gene expression can be specifically knocked down. Viral gene (including HIV) expression can be knocked down by this pathway. Due to the high frequency of mutation in HIV genome, most of the interfere RNA can knock down the gene expression of specific isolates and can not be used as a universal approach in gene therapy
15 of AIDS.

Invention disclosure

The purpose of the invention is to provide a set of nucleotides for the prevention of HIV infection and treatment of AIDS.

20 The other purpose is to provide the application of the oligo-nucleotides mentioned above.

For the purposes, following approaches were employed.

A set of RNA sequences shown thereafter, or any fragments from the sequences, which demonstrate anti-HIV infection activity and be employed in prevention and
25 treatment of AIDS. The nucleotides include single strand RNA, any fragment derived from the sequences, or double strand RNA derived by annealing of the sequences with its complements sequences.

- (1) aucaaugaggaagcugcagaaugg;
- (2) gggaagugacauagcaggaacuacuag;
- 30 (3) uaaauaaaauaguaagaauguauagcccu;

- (4) uaugggguaccugugugga;
- (5) gccaaauucccauacauuauugugc;
- (6) uuaaaugggcagucuagcagaa;
- (7) accacacacaaggcuacuucccugau;
- 5 (8) acagccgccuagcauuucaucac;
- (9) ggaugugugcuucaagcuaguaccaguu.

In the invention, conserved oligo-nucleotides sequences among all the HIV genome published were obtained by homology alignment. HIV gene expression could be knocked down and HIV genome can be degraded when the RNA was
10 introduced into mammalian cells. Pharmaceuticals derived from the conserved sequences can significantly decrease the drug resistant problems resulted from genomic mutagenesis.

A set of RNA sequences, which may be modified by other nucleotide at the 5' or 3' terminal. Usually UU were added at the 3' end of the RNA
15 fragment to assure the match between RNA with targeted mRNA.

A set of hairpin RNA sequences for the control of HIV infection and for the prevention and treatment of AIDS, the hairpin sequences were derived by the hybridization of the sequences (SEQ ID No.1 ~SEQ ID No.9) or the relevant segments at 5' terminal with their complement sequences, in which RNA
20 sequences and the complement sequences were linked by a non complement sequence. Hairpin-like RNA retains activity of RNA interference, and is particular employed to express interfere RNA in the cell since it is a RNA molecular.

A set of DNA sequences or their fragments which is against HIV infection and be used in the prevention and treatment of AIDS:

- 25 1) The DNA sequences or their fragments, which correspond to the RNA sequences shown above or their fragments (SEQ ID No.1~SEQ ID No.9 in table 1) ;or correspond to the double strand RNA sequence formed by hybridization of RNAs shown above with its complement sequence, or,
- 2) The DNA sequences or their fragments, which correspond to the RNA sequences
30 described in 1) or to their fragments which were modified at their 5' or 3' by adding nucleotides; or

3) A single strand or double strand DNA sequence, which correspond to the hairpin like RNA sequence as described above.

A set of expression vectors including both DNA vectors and RNA vectors against HIV infection and used for the prevention or treatment of AIDS, in which RNA or DNA sequences described above were contained. Interfere RNA can be expressed when the vectors containing the DNA and RNA sequences mentioned above were introduced into cells under the control of regulatory elements. The vectors include RNA vectors and DNA vectors. RNA vectors include but is not limited to retroviral vector, DNA vectors carrying DNA sequences indicated and control elements include Plasmid and viral vectors such as adenovirus associated virus (AAV).

A set of liposomes against HIV infection and for the prevention and treatment of AIDS, in which RNA, DNA sequences as well as the expression vectors indicated above against HIV infection and for AIDS treatment and prevention were coated. Interfere RNA or vectors expressing interfere RNA was introduced into cell by the liposome indicated above.

The approach to fight against HIV infection and for AIDS prevention and treatment, by which the above indicated RNAs, DNAs, expression vectors or liposomes were introduced into eukaryotic cell lines, animals or human beings. E.g. Approaches employing liposome and viral vectors.

The application of the nucleotides in the prevention of HIV infection and AIDS treatment. Pharmaceuticals for diagnosis, prevention and treatment of HIV infection and AIDS were derived from the above mentioned RNAs, DNAs, Expression vectors, liposomes or approaches.

Descriptions of the appendix figures

Fig. 1. Construction of report plasmid pEGFP-gp120.

Fig. 2 EGFP-gp120 expression was knocked down by double strand interfere RNA.

Fig. 3 EGFP-gp120 expression was knocked down by double strand

interfere RNA as demonstrated by Western-Blot.

Fig. 4 The construction of p-H1-gp120i from which the hairpin RNA could be expressed in the cells.

Fig. 5 Construction of plasmid pAAV-120i.

5 图 6 GFP-GP120 expression was knocked down by hairpin-like double strand RNA expressed by recombinant AAV.

Best approaches to realize the invention

All the protocols are generally based on the protocols described in
10 Molecular Cloning, 3rd edition.

Example 1: Most conserved HIV RNA Sequence:

HIV genome sequences published were selected and separated into 70nt fragments based on functional genes of HIV. Homology of every fragment with more
15 than 140,000 sequences in Genbank (National Center of Biological Information, USA), EMBL (Nucleotide Sequence Database in Europe Molecular Biology Laboratory), DDBJ (Japan nucleotide database) and GDB (gene database) was analyzed by BlastN 2.2.4/2.2.5. The conserved RNA sequences were selected by the following criteria: (1) The sequence is equal or longer than 19nt; (2)
20 The sequence was 100% homology with at least 1000 HIV sequences in the database; (3) If 100% homology fragments can not be found, The sequences containing 1 mismatched nucleotide were included. The results of the analysis were shown in table 1 and table 2.

Table 1. Most conserved HIV RNA sequences found by homology analysis

No	HIV gene	RNA sequence
1	gag-pol	Aucaaugaggaagcugcagaaugg
2	gag-pol	Gggaagugacauagcaggaacuacuag
3	gag-pol	uaaauaaaauaguaagauguauagcccu
4	env	Uaugggguaccugugugga
5	env	Gccaauucccauacauuauugugc

6	Env	Uuaauggcagucuagcagaa
7	Nef	Accacacacaaggcuacuucccugau
8	3-UTR	Acagccgccuagcauuucaucac
9	LTR	Ggauggugcuucaagcuaguaccaguu

Table 2. Homology analysis of the conserved RNA sequences with sequences in database

No	HIV gene	Fragment size (nt)	HIV sequence compared	100% homology sequences	Sequence (s) with 1nt mismatch
1	Gag-pol	24	1050	1050	0
2	Gag-pol	27	1051	1050	1
3	Gag-pol	29	1050	1048	2
4	env	19	1050	1050	0
5	env	24	1050	1050	0
6	env	21	1050	1050	0
7	nef	26	1082	1082	0
8	3-UTR	23	1070	1070	0
9	LTR	27	1069	1069	0

5

Example 2. HIV env gene expression was knocked down by chemically synthesized double strand RNA. .

Positive and negative (complement strand) RNA strand were synthesized according to the SEQ ID#1 with UU modification at 3' of the sequences.

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5' uaugggguaccuguguggauu

3' uuauaccccauggacacaccu

As showed in figure 1, plasmid pEGFPC1 (Clontech, CA) was double digested with EcoRI and BamHI at 37°C for 1 hour. Large fragment was extracted and

was used as vector; HIV gp120 gene was obtained by PCR using 2ng HIV cDNA (Bru strain) as template plus gp120 primers (A: 5' cgggaattctaaagagcacaagacagtggac, B: 5' cggatcctactctaccgtcagcgctcattga 100ng each) in a buffer containing 2.5u Pfu high fidelity DNA polymerase, dNTP 250μmol/L, 2.5mmol/L MgCl₂, 25mmol/L TrisHCl (pH8.3). Polymerase chain reaction (PCR) was carried out using Perkin Elmer 9700 thermocycler (94°C 30s, 50°C 30s, 72°C 90s, 30cycles), DNA fragment resulted PCR was double digested by EcoRI and BamHI (Biolabs) after being purified by Qiagen Gel Extraction Kit and ligated with the vector described above. The ligated mixture was transformed into E.coli JM109 (Promega), and the plasmid pEGFP-gp120 was obtained. Fusion protein of GFP and HIV gp120 should be expressed by transfection of the plasmid into mammalian cells.

HEK 293 cells (from ATCC) were co-transfected with 1μg plasmid pEGFP-gp120 and 1μg double strand RNA described above using LIPOFECTamine (rf. Manul from Invitrogen), The cells were assayed by fluorescent microscopy and the cell lysate were analyzed by immuno-blotting with anti-GFP antibody (Clontech) 36h after transfection. A mock double strand RNA (rf. Ds RNA correspond HIV GAG gene, see EXAPMLE 3) was employed as control.

Results: As shown in figure 2, expression of the fusion protein was knocked down by env specific double strand RNA compared to the control. The experiment was repeated twice, and was shown as DsRNA1 and DsRNA2 respectively. As shown in figure 3, the expression level of GFP-HIV GP120 fusion protein was knocked down up to 80%.

EXPAMLE 3 HIV gag gene expression was knocked down by synthesized double strand RNA

Based on the conserved gag RNA sequence (Seq ID#2 in table 1), a 21nt oligonucleotides and its complement sequence was synthesized. The sequences contain 19nt from Seq ID#2 and two U at 3' of each fragment. Double strand RNA was obtained by annealing.

5' gugacauagcaggaacuacuu

3' uucacuguaucguccuugaug

Gag gene from HIV (LAV-1, Bru isolate) was amplified and cloned into pEGFP C1 vector (Clontech, CA) as described in EXAMPLE 2, GFP-HIV gag fusion protein
5 was expected to be expressed by the plasmid when it was transfected into cells.

The plasmid as well as double strand RNA was co-transfected into HEK 293 cells by LIPOFECTamine protocol, GFP-HIV gag protein was demonstrated to be knocked down by the double strand RNA compared to the mock double strand, as shown by the fluorescent microscopy of the cells 36h after transfection.

10

EXAMPLE 4. Nef gene expression was knocked down by synthesized double strand RNA.

According to the conserved nef sequence (SEQ ID#7 in table 1), a 21nt oligo-nucleotide was synthesized with its complement RNA sequence, in which
15 the 5' 19 nt was derived from SEQ ID#7 and two U was added to the 3' of each oligo-nucleotide. Double strand RNA was obtained by annealing.

5' accacacacaaggcuacuuuu

3' uuugguguguguuccgaugaa

Gene encoding nef protein was amplified and cloned into pEGFPC1 as shown
20 in example 2, and the GFP-Nef fusion protein was expected to be expressed by the cells containing the recombinant plasmid.

HEK 293 cells were co-transfected with the plasmid obtained and the double strand RNA synthesized, it has demonstrated that the expression of the GFP-HIV nef fusion protein was knocked down by the nef specific double
25 strand RNA as compared to the mock double strand RNA, as shown by fluorescent microscopy 36hours after transfection.

EXAMPLE 5. Expression of other HIV proteins could be knocked down by synthesized double strand RNA (Table 3).

30 Table 3 Expression of other HIV genes were knocked down by the novel double strand

RNA

No	DsRNA	Targeted HIV gene	Efficacy of inhibition
1	5' aucaaugaggaagcugcaguu 3' uuuaguacuccuucgacguc	gag-pol	++++
2	5' guaagaugucuagcccuguu 3' uucauucuacagaucgggac	gag-pol	+++
3	5' uucccauacauuauugugcuu 3' uuaaggguauguaauaacacg	env	+++
4	5' aaauggcagucuagcagaauu 3' uuuuuaccgucagaucgucuu	env	+++

注: ++++60-80%inhibition; ++++ 80-100%inhibition.

Example 6: Expression of HIV envelope was knocked down by RNAi expressed by eukaryotic vector containing double DNA fragments encoding conserved hairpin SiRNA.

DNA corresponding to the fragment of SeqID#5 RNA sequence shown at table 1 and its hybrid sequence (bold italic) were synthesized, double strand DNA fragment was obtained by annealing. BamHI and HindIII sites were included at its 5' and 3' ,respectively. There are 9bp space between conserved sequence and its hybridization sequence. Fragment B is the complement sequence of fragment A:

A:5' gatcccc**ttcccatacattattgtgcttcaagagagcacaataatgtatgggaat**tttttgaaa

B:5' agcttttccaaaa**attcccatacattattgtgctctcttgaa**gcacaataatgtatgggaagg

As shown in figure 4, Human H1 promoter was amplified by primer 1 (5'-TAATTAATGCGGCCGCAATTCGAACGCTGACGTC-3') and primer 2 (5'-GCACTAGTAAGC TTGGATCCGTGGTCTCATAACAAGTTATAAGATTCCC-3' using 1μg human genomic DNA as templates. and cloned into AseI and XbaI sites of plasmid pEGFP (Clontech). The ligated mixture was transformed into E. coli JM109, and the recombinant plasmid pH1 was obtained. Annealed double strand DNA fragment

described was cloned into pH1 at its BamHI and HindIII sites, and a new recombinant plasmid, pH1-gp120i, was obtained. Hairpin RNA could be transcribed by RNA polymerase III in the cells harboring pH1-gp120i.

HEK293 cells were co-transfected with 4 µg pH1-gp120i plasmid (same amount of pH1 was used as control) and a plasmid expressing EGFP-HIV GP120. The differential expression of EGFP-HIV GP120 was assayed as described in Example 2. The results demonstrated that RNAi encoded by plasmid containing DNA fragment encoding hairpin RNA can effectively inhibit the expression of target HIV gene.

Example 7. Expression of HIV GP120 was knocked down by RNAi transcribed in the cells infected by adenovirus associated virus (AAV) which contain H1 promoter and the relevant DNA fragment encoding hairpin RNA as described in Example 6.

As shown in figure 5, plasmid pAAV-MCS (Stratagene) was digested with NotI and HindIII ; DNA fragment containing H1 promoter and DNA fragment encoding hairpin RNA corresponded to gp120 was obtained by digesting pH1-gp120 with NotI and HindIII. The fragment was ligated to vector by T4 DNA ligase, and plasmid pAAV-gp120i was constructed. HEK 293FT cells were co-transfected with the plasmid(4µg), helping plasmid pHelper(1µg, Stratagene) and plasmid pAAV-RC(2µg Stratagene) by LIPOFECTamine, and empty vector (pAAV-MCS) was used as control. Recombinant AAV and control AAV was harvested 48 hour after transfection.

HEK 293 cells were transfected by pEGFP-GP120(1µg) as described and infected by the recombinant AAV encoding RNAi or empty AAV, fluorescent of GFP expressed was assayed 24h after infection by fluorescent microscope.

As shown in figure 6, GFP-GP120 expression was significantly inhibited by the recombinant AAV which encoded hairpin RNA.

Industrial Applicability

The invention was superior to the current technology as shown below:

Highly conserved RNA fragments in all published HIV genome were obtained by homology analysis. Double strand RNA derived from the highly conserved RNA could effectively knock down the expression of HIV gene. HIV gene expression could also inhibited by dsRNA encoded by plasmid as well as recombinant adenovirus associated virus containing corresponded DNA sequence.

序列表

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<110> Beijing Joinn Pharmaceutical Center

<120> A Set of Oligo-Nucleotides Against HIV Infection and Its Application in the Prevention and Treatment of Acquired Immune Deficiency Syndrome

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